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THE EFFECT OF GLUCOSE 2-DEOXY-D-GLUCOSE AND INSULIN ON ESTRADIOL SECRETION BY CULTURED HUMAN TROPHOBLAST

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SUMMARY: Human trophoblasts were isolated in a monolayer cell culture and the effect of extra- and intracellular glucopenia on estradiol secretion was studied. Term placentas were dispersed by repeated short term trypsinization and $2x10^6$ cells plated in each dish. The de novo synthesis of estradiol was demonstrated by a 10 fold increase in estradiol secretion by supplementation of androstendione. Incubation with dibutyryl cyclic AMP produced a dose dependent increase in estradiol secretion. Low glucose (10 mg/dl) medium enhanced estradiol secretion when compared to a medium containing 50-100 mg/dl glucose. Intracellular glucopenia by 2-deoxyglucose produced an increase in estradiol secretion. The results indicate negative dependency of estradiol secretion by the trophoblast on intracellular glucose.

INTRODUCTION

In the last trimester of pregnancy outpouring of insulin in response to a glucose load is 1.5-2.5 times greater than under non-gravid conditions (1,2). Hormones of the placenta have been suggested as a possible cause to the enhanced secretion and diminished effectiveness of insulin in pregnancy. The progressive increase in plasma progesterone, estrogen and placental lactogen during the course of gestation parallel the curve of gestational enhancement of insulin secretion (1). Each of these hormones has been shown to augment islet secretory responsiveness, to directly effect the metabolism of target sites and to alter sensitivity to insulin action (3-5). For all these reasons it was expected by several investigators that placental hormones would be affected by alterations in carbohydrate metabolism and by diabetes.

However, studies in normal and diabetic pregnant women failed to show such changes. In a study of 12 insulin dependent and 9

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chemical diabetic pregnant women steroid hormones levels were followed weekly throughout the last trimester (6). A wide range of serum estradiol and progesterone was found, that was not statistically different from normal.

While chemical analysis of placental hormones in maternal circulation showed little changes in diabetes, direct visualization of diabetic placentas suggest that this is not the case within the trophoblast. Electron microscopical studies revealed signs of higher level of activity in the diabetic trophoblast. It contains prominent Golgi's bodies and membrane bound osmiophyllic secretory granules, clustering of free ribosomes and large mitochondria, which indicate increased metabolic activity (7).

It was shown that while the level of serum placental lactogen did not change in gestational diabetes, hormone levels in the amniotic fluid of diabetic mothers were significantly higher than in normal controls (8). This seems to indicate that maternal circulating hormone levels bear only little relevance to intra-placental events.

To clearly understand the hormonal events within the placenta, the trophoblast needs to be isolated from maternal circulation, thus eliminating diffusion disturbances and effects of maternal metabolism and clearance.

The purpose of the present study was to isolate the human trophoblast in a monolayer cell culture and to study the effect of extra- and intracellular glucopenia and of insulin on estradiol secretion.

MATERIALS AND METHODS

Materials. Waymouth's MB 752/1 medium, with and without glucose, fetal calf serum and trypsin were purchased from Biolab, Israel. Deoxyribonuclease, 2-deoxy-d-glucose, androstendione and N6, 02 Dibutyryl adenosine 3'5'-cyclic monophosphate were purchased from Sigma Chemicals USA. Reference estradiol was purchased from Makor Chemicals Ltd, Israel, rabbit anti 17-beta-estradiol-6-BSA serum from Miles-Yeda, Israel, and (^{3}H)estradiol from New England Nuclear Corporation, USA. Glucose concentration was measured by an Ames BMI blood analyzer.

Cell culture. Placentas were aseptically obtained from term uncomplicated vaginal deliveries, and processed within 30 minutes by a modification of the technique described by Hall et al (9). Blood was drained through the umbilical vessels and collected for later use as human cord serum. The fetal membranes, large blood vessels and fibrous tissue were sterily removed, and 1 cm pieces of placental cotyledones immersed in saline. These were forcefully agitated for wash out of blood. This procedure was repeated several times until the solution remained clear. The tissue was then chopped into 1-2 mm pieces and washed twice with Eagle balanced salt solution (EBSS), containing penicillin, streptomycin and n-butyl p-hydroxybenzoate antimycotic. The tissue was immersed in 50 ml 0.25% tripsin in EBSS with 450 units of deoxyribonuclease, and stirred for 5 minutes in a 37°C incubator (10). The supernatant was collected in centrifuge tubes, containing 1 ml fetal calf serum, and the cells harvested by centrifugation at 900xg for 10 minutes. Tripsinization of the remaining tissue was repeated 5 times. For further elimination of erythrocytes the cells pellet was suspended in 15 ml lysing buffer (0.14M NH_4Cl, 0.8mM Na_3PO_4, 0.15mM K_3PO_4, 13.7mM NaCl, 0.27 KCl, pH 7.4) for 10 minutes (11). The final cell pellet was suspended in Waymouth's MB 752/1 medium, containing 10% human cord serum, penicillin streptomycin and antimycotic (12). Cells were counted by a hemocytometer and $1.5\text{-}2x10^6$ cells plated in each 60mm Petri dish. The cells were incubated for 24 hours in a 37°C incubator in 100% humidity, and gassed with 90% air and 10% CO_2.

Incubations. Twenty four hours after dispersion of cells the medium was removed and the cells washed for 5 minutes with EBSS. The test material was then added, dissolved in 3 ml Waymouth's MB 752/1 medium, containing 2.5 ug/ml androstendione, as an estradiol precursor. The appropriate vehicle was added to control dishes. All incubations were carried out in triplicate dishes for 4 or for 16 hours. At the end of the incubation period the medium was collected and stored at -20°C for later determination of estradiol. Experiments were repeated with cells derived from 3 placentas.

Radioimmunoassay. A sample of 0.2 ml medium was extracted by 10 ml diethylether. From the organic phase 0.5 ml was transferred into glass reaction tubes and evaporated by nitrogen at 37°C. Rabbit anti estradiol antibody, containing gelatin, and (^3H)-estradiol were then added in 4°C. After an overnight incubation at 4°C the bound hormone was separated by dextran coated charcoal, and counted.

RESULTS

Microscopic observations at the end of cells dispersion were in agreement with those of previous reports (9). The culture was dominated by cytotrophoblasts. The polynucleated syncytium constituted only a minor fraction of the cells. Erythrocytes survived the procedure only sporadically, and were washed before all experiments. The number of fibroblasts was low.

The effect of androstendione. These preliminary experiments were aimed at the performance of further experiments in a serum free medium. The enrichment of a serum free medium with androstendione produced a

Table 1. The effect of 2.5 ug/ml androstendione ($^4\Delta$) and of 0.5 mM cyclic AMP (cAMP) on estradiol secretion (ng/ml) by cultured term placenta cells. Each figure represents a mean of 3 dishes, containing $2x10^6$ cells, and the SEM (in parentheses) after 4 and 16 hours of incubation

Time of incubation	-	$^4\Delta$	$^4\Delta$ + cAMP
4 hrs.	0.22 (0.02)	2.5* (0.17)	6.2* (0.50)
16 hrs.	0.76 (0.07)	7.6* (0.32)	18.9* (0.53)

*Significant at $p < 0.001$.

significant increase in the secretion of estradiol (Table I). In all subsequent experiments the culture medium was enriched with 2.5 ug/ml androstendione.

The effect of cyclic AMP. To demonstrate the functional integrity of the cultured trophoblast incubation of the cells with dibutyryl 3'5'-cyclic AMP was performed. A dose dependent stimulation of estradiol secretion was observed (Figure 1).

The effect of low-glucose medium. The cells were incubated with medium containing 10 to 100 mg/dl of glucose. Equal osmolality in all the media was assured by modification of the concentration of urea.

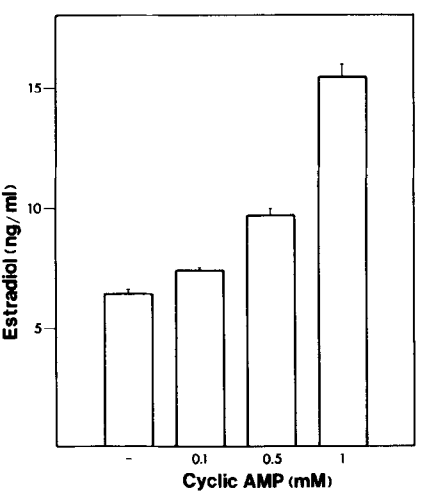

Figure 1: The effect of dibutyryl cyclic AMP on estradiol secretion by cultured trophoblast. Bars represent the mean and SEM of 3 dishes, containing $2x10^6$ cell, incubated for 4 hours with cyclic AMP.

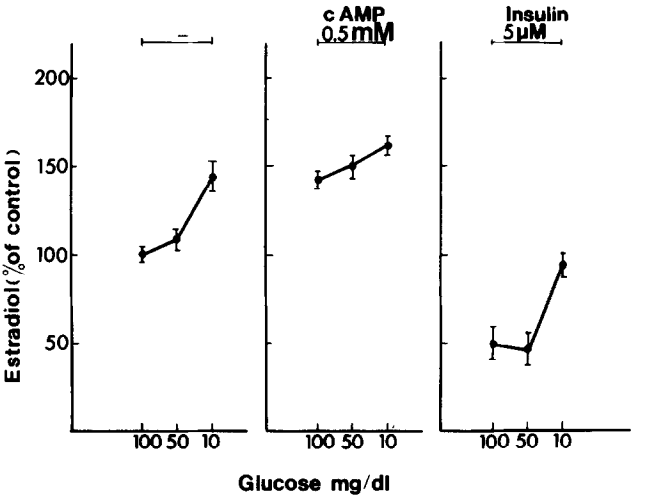

Figure 2: The effect of medium containing 10-100 mg/dl glucose on estradiol secretion by cultured trophoblast. Each point represents the mean and SEM of 2 experiments with different placentas. In each experiment 3 dishes were incubated for 4 hours and the medium assayed for estradiol values were adjusted to a 100% secretion by cells incubated with 100 mg/dl glucose. Similar incubations were carried out with 0.5 mM dibutyryl cyclic AMP and 5 µM insulin.

Measurement of osmolality by a cryoosmometer revealed variations of 325 ± 4 mOsm/kg. A significant increase in estradiol secretion was observed with the 10 mg/dl glucose medium, compared to the 50-100 mg/dl glucose media (Figure 2). Similar increase was observed in the presence of 0.5 mM dibutyryl cyclic AMP or by 5 uM of insulin.

Insulin effect. Insulin produced a dose dependent decline in estradiol secretion by the cultured trophoblast (Figure 3).

In determining insulin concentration in the culture medium before incubation and 4 hours later we have shown that by addition of 0.25% bovine serum albumin to the culture medium, as suggested by Steel et al (13), degradation of insulin was insignificant.

2-deoxy-d-glucose (2DG). When 5 mM 2DG, a competitive inhibitor of glucose transport and metabolism, was added to the culture medium, a significant increase in estradiol secretion was observed both in a low-glucose medium and in a medium containing 500 mg/dl of glucose (Table II).

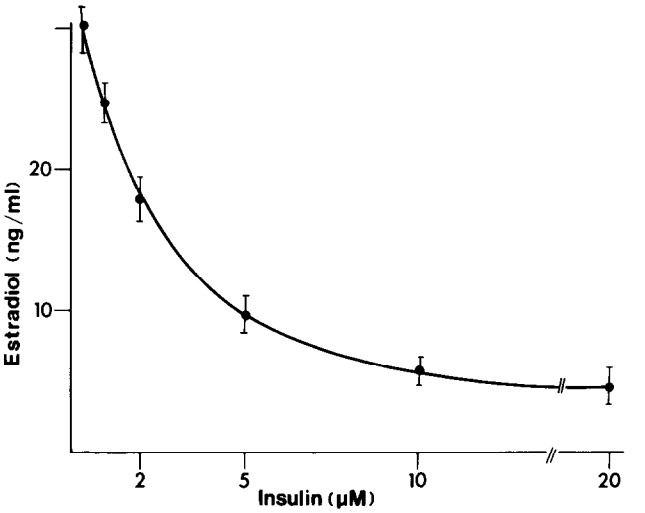

Figure 3: The effect of insulin on estradiol secretion by trophoblast cultured for 4 hours in a medium containing 500 mg/dl glucose.

DISCUSSION

Enzymatic dispersion of normal human trophoblast, and maintenance of trophoblast in a monolayer cell culture, have been previously employed by several investigators (9-11). By a slight modification of the method of Hall et al (9) we have been able to disperse human placenta and to maintain a functioning trophoblast for several days.

In an attempt to perform experimental incubations in a serum free medium it was supplemented with androstendione, as a precursor for estradiol. Moreover, the increased level of estradiol in the presence

Table 2. The effect of 0.5 mM cyclic AMP (cAMP) and 5 mM 2-deoxy-d-glucose (2-DG) on estradiol secretion (ng/ml) by cultured term placenta cells. The medium contained 10 or 500 mg/dl of glucose. Equal osmolality was assured by addition of urea to the low-glucose medium. All dishes contained 2.5 ug/ml androstendione. Each figure represents a mean of 3 dishes, containing $2x10^6$ cells, and the SEM (in parentheses).

Glucose	-	cAMP	cAMP + 2-DG
10 mg/dl	4.6 (0.8)	15.7* (0.63)	18.3* (1.0)
500 mg/dl	4.7 (1.28)	8.5* (1.0)	13.9* (0.9)

*Significant at $p < 0.01$.

of its precursor provides proof of actual biosynthesis in the cultured trophoblast, rather than release of preformed hormone. The functional integrity of the cultured trophoblast was also demonstrated by the dose dependent increase in estradiol secretion to the addition of dibutyryl cyclic AMP.

From the results it is clear that incubation of the cells in low-glucose culture medium increases estradiol secretion by the cultured trophoblast. Similar to the effect of a low-glucose medium, an intracellular glucopenia, produced by the addition of the glucose metabolism competitive inhibitor 2-deoxyglucose, along with cyclic AMP, induced a significant increase in estradiol secretion by the cells. Insulin produced dose dependent decrease in estradiol secretion.

It is suggested that within the trophoblast a regulation mechanism exists, in which estradiol produces insulin resistance (3), and this, by lowering intracellular glucose concentration and utilization, stimulates estradiol secretion. When insulin resistance is overcome by increased concentrations of insulin, transport of glucose into the cells and glucose metabolism is enhanced, whereby estradiol secretion is inhibited.

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